COMBINED EXPOSURE TO HALOTHANE AND 1 OR 2 Gy IONIZING RADIATION CAUSES A SYNERGISTIC EFFECT IN DNA DAMAGE IN THE BLOOD AND LIVER OF SWISS ALBINO MICE

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Abstract. Patient immobilization by general volatile anesthesia (VA) during medical radiology treatment is sometimes necessary and annual trends are increasing. Ionizing radiation (IR) exposure is known to cause some level of DNA damage since IR is a well-known genotoxic and cytotoxic agent, although the doses used are kept to a minimum, with good localization in order to protect as much healthy tissue and organs as possible from exposure. Recently, a growing number of studies have demonstrated that volatile anesthetics can also cause DNA damage effects in patients, and in occupationally exposed personnel. Since there are no studies on the combined effects of IR and VA, we decided to use an animal model of Swiss albino mice to determine whether there are elevated levels of DNA damage after combined exposure by mimicking real conditions of exposure during radiology treatment. Healthy male mice (5 animals per group) were anesthetized by inhaling 2.4% halothane for 2 hours and then were exposed to either 1 or 2 Gy of ionizing radiation (¹⁰⁰Co source). Groups were examined immediately after exposure, and again after 2, 6 and 24 hours. Blood was taken from the tail, and liver after animal sacrifice. The study was approved by the Ethics Committee of the Faculty of Science, University of Zagreb, Croatia, and designed in accordance with the relevant Croatian guidelines (Animal Protection Act, Ordinance on the protection of animals used for scientific purposes). Duplicate samples were prepared for the alkaline comet assay, and DNA damage of a total of 200 comets per point was assessed with Comet Assay IV software. The results demonstrated that both halothane and IR caused elevated DNA damage levels, and when applied in combined treatment caused synergistic effect additional damaging effect that was not repaired even 24 hours after exposure. These data confirm concerns about the safety of combined VA and IR exposure, and indicate the need for further investigation on the safety and proper use of the type of anesthetic needed during radiotherapy.

Key words: Halothane, ionizing radiation, comet assay, cellular DNA repair index

1. INTRODUCTION

Since ionizing radiation (IR) is a well-known genotoxic and cytotoxic agent [1], overall exposure, dose and the number of exposures should be kept to a minimum. According to the International Agency for Research on Cancer (IARC) and World Health Organization (WHO), the predictions for 2025 are that up to 2 million of newly diagnosed cancer patients in European countries will require radiotherapy treatment [2]. Treatment planning with precision irradiation of the affected body part and the use of novel irradiation techniques [3], [4] in different types of radiotherapy (intraoperative radiotherapy, brachytherapy, fractionated and hypofractionated radiotherapy), have demonstrated that it is possible to administer less radiation and patients subsequently experience fewer side effects [5]. The usual dose in tumor treatment received during one fraction in conventional radiotherapy or in hypofractionated doses is 2 or 1 Gy [6]. In pediatric cancer patients and cancer patients with anxiety problems and/or claustrophobia, site precision and dose received can also be improved by patient immobilization [6], with the help of general anesthesia [7], [8]. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a non-flammable, halogenated general anesthetic administered by inhalation. It provides fast induction of anesthesia by depressing the central nervous system, thereby producing a reversible loss of consciousness and sensation [9], [10]. Volatile anesthetics with a similar mechanism of action (enhancing the inhibitory postsynaptic channel activity (gamma-aminobutyric acid (GABA) and glycine) and inhibition of the excitatory synaptic channel activity (N-methyl-D-aspartate (NMDA), nicotinic acetylcholine, serotonin, and glutamate) in the central nervous system are considered generally safe for patients [10]. Some studies have demonstrated different side effects among patients, especially children (depending on the type of the anesthetic used), and among occupationally exposed personnel [11] - [13]. The toxic effect depends on the dose and number of exposures, metabolism and toxicokinetics of anesthetics in the liver, kidney or brain [11] - [14].

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determine whether the levels of DNA damage are elevated after combined exposure.

2. MATERIALS AND METHODS

2.1. Animals and ethical approval

In the breeding unit of the Department of Biology, Faculty of Science, University of Zagreb, Croatia, Swiss albino male mice were housed in standard breeding conditions (22±1°C, humidity 50-70%, 12-hour photoperiod, fed with standard laboratory diet (Standard Diet GLP, 4RF1, Mucedola, Settimo Milanese MI, Italy), with water ad libitum). The study was approved by the Ethics Committee of the Faculty of Science (University of Zagreb, Croatia; No. 251-58-508-11-9) and designed in accordance with relevant Croatian guidelines: Animal Protection Act [15], Ordinance on the protection of animals used for scientific purposes [16], and the relevant EU Directive [17].

2.2. Chemicals

Unless otherwise specified, chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Inhalation anesthetics halothane (Halothane®) was provided by Abbott Laboratories LTD (Queensborough, UK).

2.3. Anesthesia and irradiation

Mice with a body weight of 20-25 grams (age 60±5 days), were randomly divided into 24 groups (each of 5 animals): four groups only anesthetized with halothane, eight groups only exposed to IR (four groups to 1 Gy, four groups to 2 Gy), eight groups anesthetized with halothane and exposed to IR (four groups to 1 Gy, four groups to 2 Gy, and four control groups that received no treatment.

Except for the control and only IR groups, mice were anesthetized in an induction chamber connected to an anesthetic machine (Sulla 800; Dräger) with a compatible evaporator in which 2.4 vol % halothane was mixed with a 50:50 mixture of oxygen and air (3 L/min) in continuous flow for 2 h. Acceptable deep anesthesia was achieved when mice were sleeping calmly, breathing spontaneously, not wiggling the tail, which corresponds to classified stage 3 – Surgical Anesthesia [18], [19].

After 2 hours of anesthesia, mice included in groups for combined treatment, were irradiated with 1 Gy or 2 Gy γ-radiation at a dose rate of 0.88 Gy/min (86Co source, Theratron Phoenix teletherapy unit, Atomic Energy Ltd., at the Sveti Duš Clinical Hospital). The IR only groups received the same treatment.

2.4. Sacrifice, sampling, alkaline comet assay

Animals were sacrificed by cervical dislocation in compliance with the laboratory animal legislation [15], [16]. Five microliters of blood were taken from the mouse tail, while small pieces of freshly resected mice liver tissue were mechanically homogenized using chilled homogenization buffer (0.075 M NaCl and 0.024 M Na₂EDTA, kept at 4°C, freshly prepared, at a ratio of 1 g tissue to 1 mL buffer. The single cell suspension homogenate at 4°C was immersed into agarose gel immediately after sample preparation.

The alkaline comet assay or single cell gel electrophoresis assay was carried out under alkaline conditions in standardized conditions, as described [19]. Either 5 µL of blood or 10 µL of single liver cell suspension were mixed with 100 µL of 0.5% low melting point (LMP) agarose (freshly prepared, kept at 37°C in a water bath) and layered above and covered with coverslips (Biognost, Zagreb, Croatia). Slides were kept horizontally for solidification at 4°C for 10 minutes and following this another layer of 0.5% LMP agarose was applied. After solidification and coverslip removal, slides were immersed for two hours at 4°C in a freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1 % sodium saccharinate, pH 10, with 1% Triton X-100 and 10% dimethyl sulfoxide (Kemika, Zagreb, Croatia)). Denaturation and electrophoresis were carried out at 4°C under dimmed light in a freshly prepared denaturation solution (300 mM NaOH, 1 mM Na₂EDTA, pH 13). After 20 min of denaturation, electrophoresis was carried out for 20 min at 25 V (300 mA, 0.8V/cm). After electrophoresis, slides were neutralized three times at five-minute intervals by adding 0.4 M Tris-HCl buffer, pH 7.5. Images of 40 randomly selected cells were analyzed for each sample (total of 200 comets for each group analyzed). After staining with ethidium bromide (20 µg/mL, 10 minutes), slides were examined using an epifluorescence microscope (Olympus BX41, Tokyo, Japan, 200x magnification) with a CCD camera with a computer-based image analysis system (Comet Assay IV software, Instem, London, UK). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets. DNA damage was determined as tail intensity parameter (TI, percentage of genomic DNA that migrated during the electrophoresis from the nuclear core to the tail).

2.5. Cellular DNA repair index

The cellular DNA repair index (CRI) was calculated from the medians of the TI parameter according to the formula by Nair & Nair [20]. The CRI serves to quantify the cells’ efficiency to repair and re-join strand breaks, and to determine whether treatment had an influence on faster, slower or delayed repair compared to the control.

2.6. Statistical analysis

Statistica 9.0 (Statsoft, Tulsa, USA) and Statistica 13.5.0.17 (TIBCO Software Inc., California, Palo Alto, USA) was used for statistical analysis. Descriptive statistics of tail intensity parameter was calculated for mean, median, standard error (SE) and standard deviation (SD). The data were statistically compared using Mann Whitney U-test. The level of statistical significance was set at P<0.05 or even less.

3. Results

In blood cells, halothane induced statistically increased DNA damage compared to the control at 2, 6
and 24 h after treatment, both alone and in combination with 1 Gy irradiation, while in combination with 2 Gy, statistically higher DNA damage was observed immediately after, 6 and 24 h after treatment (Figure 1).

In liver cells, increased DNA damage was observed only 6 h and 24 h after treatment of non-irradiated animals and after 6 h of the combined treatment with 2 Gy irradiation (Figure 2). Nuclei after the alkaline comet assay in blood and liver cells are presented on Figure 3. Halothane blocked DNA repair after 2 h in blood cells (Figure 4).

4. CONCLUSION
Both halothane and IR demonstrated elevated levels of DNA damage, with combined treatment causing an IR dose-dependent synergistic damaging effect that was not repaired even 24 hours after the exposure. These data confirm previous concerns about safety of combined VA and IR exposure, and indicate the need for further investigation regarding the safe and proper use of anesthetic during radiotherapy.

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